Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium

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The mammary glands of prepubertal estrogen receptor (ER)$\beta-/-$ mice are morphologically indistinguishable from those of WT littermates. It appears that, although ERβ is expressed in the mouse mammary gland, it is not involved in ductal growth of the gland. In this study, we examined the possibility that ERβ has a role in the differentiated function of the mammary gland. Pregnancy is rare in ER$\beta-/-$ mice, but an intensive breeding program produced seven pregnant ER$\beta-/-$ mice, of which five did not eat their offspring and continued to successful lactation. Histomorphological comparison of lactating glands revealed that alveoli were larger and there was less secretory epithelium in ER$\beta-/-$ than in WT mice. Ultrastructural analysis showed abundant milk droplets and normal apical villi in the luminal epithelial cells, but the extracellular matrix and lamina basalis were reduced, and very frequently the interepithelial cell space was increased. Levels of the adhesion molecules, E-cadherin, connexin 32, occludin, and integrin α2 were reduced, and no zona occludens was detectable. In addition, there was widespread expression of the proliferation marker, Ki-67, in luminal epithelial cells in ER$\beta-/-$ but not in WT mice. These findings suggest a role for ERβ in organization and adhesion of epithelial cells and hence for differentiated tissue morphology. We speculate that, because a reduced risk for breast cancer is conferred on women who breast-feed at an early age, ERβ could contribute to this risk reduction by facilitating terminal differentiation of the mammary gland.

lactation | cadherin | integrin | tight junction

Targeted disruption of estrogen receptor (ER)β in mice has revealed that this is a functional receptor in both males and females and that, in addition to its role in reproductive functions (1), it is also important in the cardiovascular (2) and central nervous systems (3). Female mice homozygous for the mutated ERβ gene (ER$\beta-/-$ mice) have been described as subfertile or infertile (4, 5). The ovarian defect is due to early atresia of antral follicles and failure to ovulate. As a result, corpora lutea are rare in ER$\beta-/-$ mouse ovaries (6).

Prepubertal ER$\beta-/-$ females appear to have a normal mammary histology (5) with unaffected ductal outgrowth of the mammary gland anlage. However, because corpora lutea are rare, little progesterone is produced in the ovaries and, in contrast to their WT littermates, ER$\beta-/-$ mammary glands fail to develop ductal side branches and alveoli after puberty. On administration of progesterone, side branching occurs, and mammary glands of ER$\beta-/-$ mice appear morphologically indistinguishable from those of their WT littermates (7). As judged by nursing behavior and growth of their pups, ER$\beta-/-$ mice have previously been reported to lactate effectively (5). In contrast, in female mice lacking aromatase (8) or ERα (9), mammary glands fail to develop beyond the prepubertal stage. From these studies, it has been concluded (5) that estrogen, acting through ERα but not through ERβ, is essential for normal mammary gland development and function.

ERβ plays a multifaceted role in functional differentiation of various epithelial and nonepithelial cell types (10). From the high level of expression of ERβ in the rodent mammary gland (11), it is logical to assume that this receptor should have a function. Because it does not appear to be essential for prepubertal growth, we have examined the possibility that it is important for terminal differentiation of the gland. We analyzed cytoarchitecture and markers of differentiation such as occludin and connexin (Cx)-32 in mammary glands of WT and ER$\beta-/-$ mice during lactogenesis and lactation and demonstrate here that ERβ signaling is required for normal lobuloalveolar development.

Methods

Animals and Collection of Tissues. WT and ER$\beta-/-$ mice were bred at our ER$\beta-/-$ mouse colony at the animal facility at the University of Turku, Turku, Finland, and were housed under 12:12-h light–dark cycle at 21°C with 50% humidity with free access to water and a soy-free standard chow diet (SDS, Whitham, Essex, U.K.). At ≈8 wk of age, ER$\beta-/-$ mice were set for breeding with ER$\beta\pm$ males. Genotyping by PCR was performed as described (12). Mice were killed by CO_2 asphyxiation. Thoracic and inguinal mammary glands were excised. One inguinal gland was frozen immediately and stored at −80°C until used for immunohistochemistry, RT-PCR, and Western blotting. The second inguinal gland was fixed in 4% paraformaldehyde overnight and routinely embedded in paraffin wax. Thoracic/inguinal glands were prepared as whole mounts (see below).

Preparation of the Mammary Gland Whole Mounts. Mammary gland whole mounts were prepared as described by Rasmussen *et al.* (13). Briefly, mammary glands were excised from mice and fixed on glass slides in Carnoy's fixative (formula 1: glacial acetic acid–absolute ethanol). They were then hydrated in 70% ethanol, rinsed in water, and stained overnight in carmine alum followed by dehydration in increasing series of alcohol and clearing in xylene. The glands were mounted with cover slips with Permount (Fisher; YA-Kemia Oy, Helsinki).

Histology. After paraffin embedding, sections (4 μm) were mounted on organosilane-coated slides. Hematoxylin/eosin staining was used for histological evaluation under the light microscope. Values obtained for histological evaluation are based on simple point-counting morphometry.

Immunohistochemistry. Antibodies were purchased from Santa Cruz Biotechnology (sc), unless otherwise indicated. The cellular presence of E-cadherin (sc-7870, 1:100), N-cadherin (sc-1502, 1:100), cadherin 11 (sc-6463, 1:100), pan-cadherin (sc-10733, 1:100), β-catenin (sc-1496, 1:100), integrin α2 (VLA-2α, Transduction Laboratories, Lexington, KY, 1:100), integrin α6 (sc-

Abbreviations: ER, estrogen receptor; Cx, connexin; sc, Santa Cruz Biotechnology.

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6597, 1:100), and Cx-32 (Des5, 1:100 and 2A, 1:100) (14) was detected in paraffin sections by standard immunofluorescence procedures adapted from Becker *et al.* (14). Sections were incubated with primary antibodies [in 3% (wt/vol) BSA in PBS] overnight at 4°C. PBS was used in place of primary antibodies in negative controls. Slides were washed with PBS and incubated with the respective FITC-conjugated donkey secondary antibodies (Jackson ImmunoResearch) for 45 min at 37°C. Nuclei were counterstained with propidium iodide in the case of Cx-32 stains. For occludin staining, frozen sections of 9-μm thickness were air dried for 30 min. After washing with ice-cold methanol and acetone each for 3 min, sections were fixed for 10 min at room temperature in 4% paraformaldehyde. All sections were treated with 0.1% Triton X-100 in PBS for 5 min, incubated with 10% normal donkey serum in PBS for 30 min at 25°C to reduce unspecific binding of secondary antibody, and exposed in sequence to antibodies against occludin (rabbit polyclonal, Zymed, 1:100) and FITC-anti-rabbit conjugate. After incubation, sections were mounted in Vectashield antifading medium (Vector Laboratories) and examined at comparable depths on a Leica TCSSP confocal microscope (Leica, Heidelberg, Germany). At $\times$63 magnification used to evaluate Cx expression, each fluorescent spot indicates a gap junction plaque (see ref. 14).

For α-smooth muscle actin and Ki-67 staining, after antigen retrieval, paraffin sections were dewaxed in xylene, rehydrated, and then processed as previously described (3). Sections were then incubated overnight at 4°C with mouse monoclonal anti-α-smooth muscle actin antibody (clone 1A4, Sigma) or polyclonal goat-anti-Ki-67 antibody (sc-7846, 1:400) in 3% (wt/vol) BSA. For negative controls, primary antibody was replaced by 3% (wt/vol) BSA. The ABC method was used to visualize signals according to the manual provided by the manufacturer (Vector Laboratories). The stained cells were viewed under a Zeiss Axioplan2 microscope.

Construction of Figures. Each single confocal image in a z-series captures the distribution of antibody-labeled protein in a 0.5-μm section of the tissue (Fig. 3 *A–H* and Fig. 4 *D–G*). The z series was then projected to give a single image showing the distribution of the visualized protein in a tissue depth of up to 15 μm [see Becker *et al.* (14) for methods]. This provides a two-dimensional summation of the number and distribution of antibody labeled structural proteins and gap junctions in that volume of tissue.

Electron Microscopy. Mammary glands were dissected, and small pieces were cut and immediately fixed in 2% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (caco) and 0.1 M sucrose and 3 mM $CaCl_2$, pH 7.4, overnight. Specimens were rinsed in 0.15 M caco and postfixed by incubation for 2 h in 2% osmium tetroxide in 0.07 M caco containing 3 mM $CaCl_2$. The specimens were dehydrated in an ascending series of alcohol into acetone and embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, VT). Semithin sections, $\approx$0.5 μm, were placed on glass slides, stained with toluidine blue, and examined in a light microscope. Ultrathin sections were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 (FEI, Eindhoven, The Netherlands) transmission electron microscope at 80 kV.

Results

Histomorphological Analysis of Lobuloalveolar Development in ERβ−/− Mice. Whole mounts were made of glands taken from 6-mo-old virgin mice on day 15 of pregnancy or on day 6 of lactation (Fig. 1). As reported previously (5, 15), loss of ERβ did not affect ductal growth but seemed to decrease side branching (Fig. 1 *A* and *D*) in nulliparous mammary glands. Previous work has shown that on administration of progesterone, side branching occurs in mammary glands of both WT and ERβ−/− mice

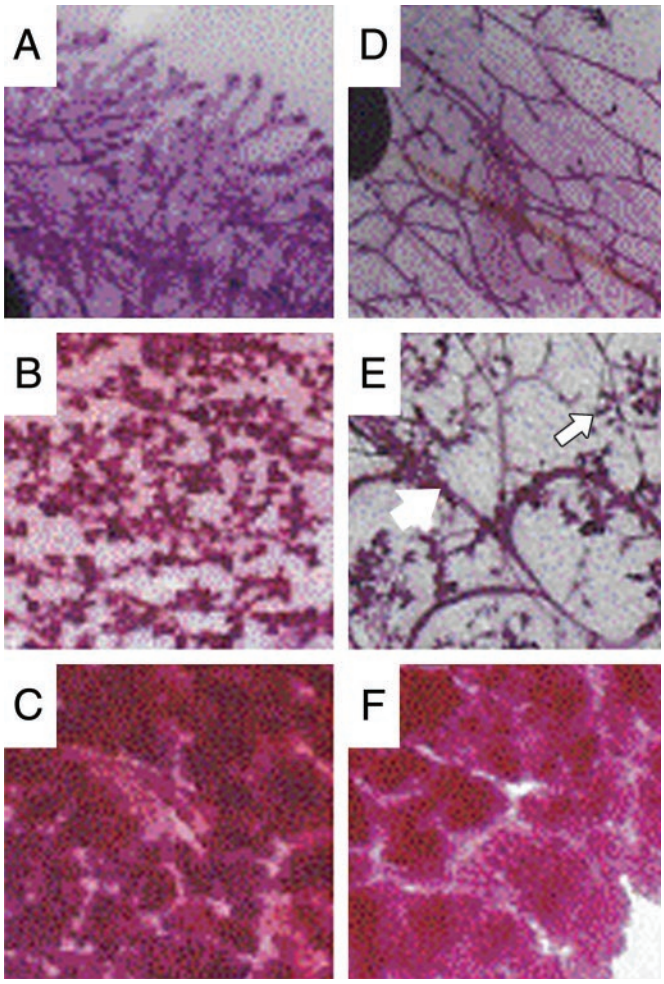

Fig. 1. Structure of normal and mutant mammary glands at different endocrine stages. Whole-gland stain of thoracic no. 3 mammary glands of (*A*) mature (4 mo) virgin; (*B*) mid-pregnant (day 15); and (*C*) lactating (day 6) WT mammary gland. Matched ERβ−/− females; (*D*) virgin (4 mo); (*E*) mid-pregnant (day 15); and (*F*) lactating (day 6), display-impaired lobuloalveolar development. The pictures are representative for the observations made in all five mice analyzed per group.

(7), revealing that in the absence of ERβ, there is a full growth response to progesterone. At mid-pregnancy, in mammary glands of WT mice, there was extensive lobuloalveolar development, with formation of acini (Fig. 1*B*). In ERβ−/− mice at this stage, there were distended ducts (Fig. 1*E*, open arrow) but lobuloalveolar development was not as extensive as in WT mice (Fig. 1*E*, filled arrow). On day 6 of lactation, in glands of WT mice there was complete lobuloalveolar development, with glands filling the fat pad (Fig. 1*C*), whereas in ERβ−/− mice, glands only partially penetrated the fat pad (Fig. 1*F*).

Gross histomorphology was examined in WT and ERβ−/− mouse mammary glands with hematoxylin/eosin-stained sections (Fig. 2 *A* and *B*). At day 6 of lactation, mammary glands of both WT and ERβ−/− mice were fully traversed by ducts, but in the ERβ−/− mice, there were abnormally large alveoli ($\approx$45% of total luminal area vs. 20% in WT mice), which had incompletely penetrated the fat pad, so that a much higher content of adipose tissue was visible ($\approx$30% in ERβ−/− mice vs. 5% in WT mice). This means that the relative amount of epithelium is lower in ERβ−/− mice. As in WT mice, the cytoplasm of ERβ−/− mouse mammary epithelium contained abundant secretory vesicles (Fig. 2 *C* and *D*).

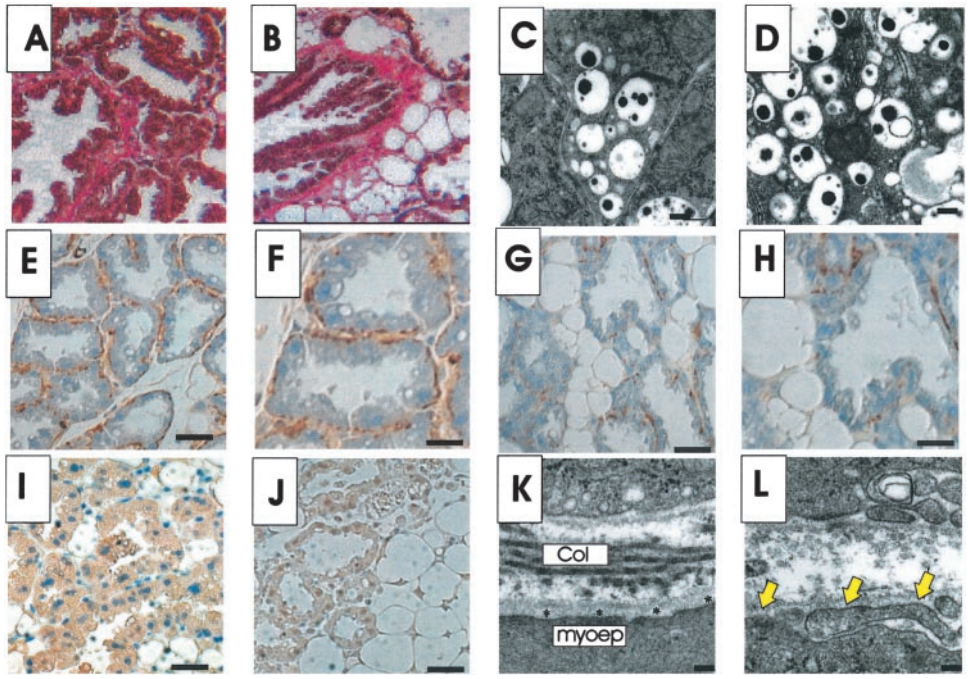

Fig. 2. Histology and ultrastructure of mammary tissue from lactating (day 6) WT and ER$\beta-/-$ mice. (*A* and *B*) Hematoxylin/eosin-stained section of lactating mammary glands. (*A*) WT (×10), (*B*) ER$\beta-/-$ (×10). In ER$\beta-/-$ mice, there is incomplete penetration of the mammary fat pad, less epithelium, and increased alveolar and ductal volume. (*C* and *D*) Electron micrograph of apical part of alveolar luminal epithelial cells. (*C*) Cytoplasm of a WT gland showing vesicles containing milk protein (dark spots). (*D*) Cytoplasm of an ER$\beta-/-$ gland showing abundant vesicles containing milk protein. (Bar = 200 nm.) (*E–H*) Immunohistochemical detection of α-smooth muscle actin on fixed sections from mammary glands of WT and ER$\beta-/-$ mice. Representative sections of WT and ER$\beta-/-$ lactating (day 6) mouse mammary glands, respectively; (*E* and *G*) low magnification; (*F* and *H*) high magnification. [Bar = 50 μm (*E* and *G*) or 25 μm (*F* and *H*), respectively.] (*I* and *J*) Immunohistochemical staining for Ki-67 in lactating WT (*I*) and ER$\beta-/-$ (*J*) mice. In mammary glands of WT mice, no Ki-67 expression is detectable (*I*). In ER$\beta-/-$ lactating mammary glands, there is Ki67 expression in the nuclei of many luminal epithelial cells (*J*) (percent positive cells: 58 ± 13%, $n = 6$). (*K* and *L*) Electron micrograph of basal part of alveolar luminal epithelial cells. The basement membrane in WT mouse shows a homogeneous layer of the lamina lucida (*) close to a myoepithelial cell (myoep) and visible collagen fibers (col) (*K*). The basement membrane in ER$\beta-/-$ mice shows a more translucent (arrows) lamina lucida. No collagen fibers are visible in the micrograph, and they are rarely found in the glands of ER$\beta-/-$ mice (*L*). (Bar = 200 nm.)

At 6 mo of age, in both WT and ER$\beta-/-$ mice, α-smooth muscle actin-positive cells were arranged in a continuous monolayer around the luminal epithelial cells (data not shown). In lactating WT animals, these alveolar myoepithelial cells showed the typical punctate appearance surrounding the alveolar luminal epithelial cells (Fig. 2 *E* and *F*). In lactating ER$\beta-/-$ mice, however, actin-containing cells were sparse, and in some alveolar units there was an almost complete lack of actin (Fig. 2 *G* and *H*). In addition, the proliferation marker Ki-67 (16) was expressed in the nuclei of the apparently fully differentiated luminal epithelial cells of ER$\beta-/-$ but not WT mice (Fig. 2 *I* and *J*). The basal villi in glands of ER$\beta-/-$ mice appeared normal but the basement membrane was thin. It was ≈30–50 nm wide in ER$\beta-/-$ mice and ≈100 nm in WT mice (Fig. 2 *K* and *L*). The inner border (laminin-containing lamina lucida) of the basement membrane was translucent and collagen fibers, which were frequent in WT mice, were rare in ER$\beta-/-$ mice.

Spatial Expression of E-Cadherin, β-Catenin, Integrin α2, and Integrin α6. In WT mice, E-cadherin-containing adherens junctions were very conspicuous on basolateral surfaces of luminal epithelial cells in ducts and alveoli in mammary glands of virgin and pregnant mice as well on lateral membranes in lactating mice (Fig. 3 *A–C*). In mammary glands of virgin ER$\beta-/-$ mice, E-cadherin expression appeared to be normal (Fig. 3*E*). However, expression was very weak in alveoli of midpregnant and lactating mice and, in several alveoli, no E-cadherin immunostaining was detected (Fig. 3 *F* and *G*). No positive staining for either N-cadherin or OB-cadherin was detected (data not shown). The expression of β-catenin was also affected by loss of ERβ but, surprisingly, in view of the decrease in expression of E-cadherin, β-catenin immunostaining was more intense on the membranes of luminal epithelial cells of lactating ER$\beta-/-$ mice (Fig. 3 *D* and *H*).

Integrin α2 was mainly confined to the basal part of the plasma membrane in the epithelium of virgin WT mice. At midpregnancy (day 15), integrin α2 levels were reduced, but expression was up-regulated by day 6 of lactation. In marked contrast, no expression of integrin α2 could be detected at any endocrine stage in mammary glands of ER$\beta-/-$ mice (Table 1). Integrin α6 expression was elevated in virgin ER$\beta-/-$ mice. It was located at the basal plasma membrane of the alveoli and in the surrounding stroma (not shown) and expression was not increased in mid-pregnant or lactating mammary glands in mice of either genotype (Table 1).

Expression of Tight Junctions and Gap Junctions. Morphologically, epithelial cells were similar in WT and ER$\beta-/-$ mice. The polarity of the epithelial cells and the number of apical villi were comparable. In sections perpendicular to the plane of the epithelium, normal tight junctions were clearly visible in WT animals. They were located at the apical surface between all of the luminal epithelial cells and were all of the same length (Fig. 4*A*). In ER$\beta-/-$ mice, irregular contact points of various lengths were frequently seen.

Their morphology varied from short punctate fusions (Fig. 4*B*) to long irregular complexes (Fig. 4*C*). Desmosomes were identified in glands of mice of both genotypes. In ER$\beta-/-$ mice, a common finding was that the intercellular distances were wider than in WT mice (Fig. 4*B*). By immunofluorescence microscopy, occludin, the major integral epithelial tight-junction protein (17), was detected in

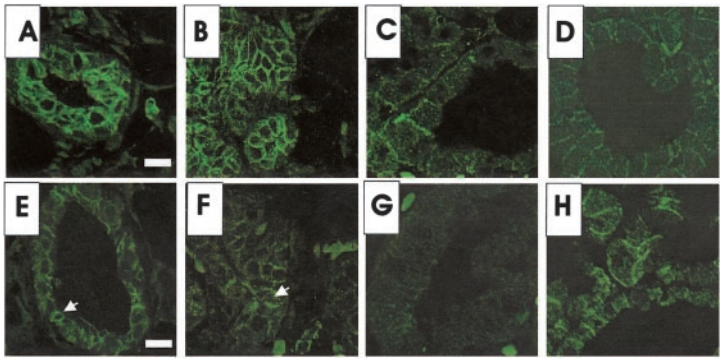

Fig. 3. Spatial expression of E-cadherin and β-catenin during mammary gland differentiation in WT and ERβ−/− mice. (*A–C* and *E–G*) Immunofluorescence labeling of E-cadherin WT (*A–C*) and ERβ−/− (*E–G*) mice; virgin (*A* and *E*), midpregnant (*B* and *F*), and lactating (*C* and *G*). Note in virgin and pregnant ERβ−/− mammary gland epithelium labeling is reduced (arrow) and appears less well organized; in lactating ERβ−/− glands, E-cadherin immunoreactivity is not detectable. (*D* and *H*) Immunofluorescence labeling of β-catenin in lactating WT (*D*) and ERβ−/− (*H*) mice. β-Catenin immunoreactivity is increased in mammary glands of ERβ−/− mice. The pictures are representative for the observations made in all of the five mice analyzed per group. (Bars = 20 μm.)

the apical part of the luminal epithelial cells in lactating WT mice (Fig. 4*D*). In lactating ERβ−/− mice, however, no positive immunostaining of occludin was detected in any of the five mouse breasts examined (Fig. 4*E*). With two different anti-Cx-32 antibodies, intense labeling was seen in the luminal epithelium of both the ducts and alveoli in lactating glands of WT mice (Fig. 4*F*). The staining, which appeared as small dots on the lateral surface of the epithelial cells was much less intense in alveoli in mammary glands of lactating ERβ−/− mice (Fig. 4*G*).

Discussion

A major part of mammary gland development occurs postnatally, with distinct periods of intensive morphogenesis taking place at puberty and during pregnancy and lactation (18). The present study has shown that the mammary gland develops normally until puberty in ERβ−/− mice, but that ERβ is essential for the complete differentiation of the gland during pregnancy and lactation. The major morphological findings in ERβ−/− mice were: incomplete penetration of the fat pad by glandular tissue; an increase in the size of the lumen of ducts and alveoli; concomitantly a reduction in the number of alveoli per area; a reduction in the content of secretory epithelium and a reduction in the width of the innermost layer (laminin containing lamina lucida) of the basement membrane. Biochemically, there was a reduction in expression of collagen in the extracellular matrix, E-cadherin, integrin α2 (the laminin receptor), occludin (the tight junction protein), Cx-32 (the gap junction protein) and smooth muscle actin (a marker of myoepithelial cells). In addition, the apparently functionally differentiated epithelium expressed Ki67 indicating that cells are not in G_0. All these changes point to a single conclusion, i.e., that the mammary gland of lactating ERβ−/− mice is less well differentiated that that of WT mice.

Both laminin and integrins are required for normal ductal morphogenesis during development. A failure in basement membrane formation, or even a dysregulation of basement membrane architecture, can lead to impaired development or developmental arrest in a given tissue (19). ERβ−/− mice lack integrin α2 subunit expression but have an elevated level of integrin α6 in their mammary glands. Streuli and Edwards (20) have provided evidence that integrin α2β1 is the receptor driving milk protein expression. In contrast, we found that in the absence of integrin α2, ERβ−/− mice produce milk and can nurse their pups. Our data suggest that integrin α6β1 could be the receptor through which laminin induces mammary gland proliferation and milk protein synthesis, whereas integrin α2 might be involved in maintaining differentiated morphology. This notion is supported by the observation that the mammary glands of integrin α2−/− mice show no morphological or histological changes and these mice lactate normally (21).

Table 1. Integrin α2 and α6 expression in WT and ERβ−/− mammary glands

	WT			ERβ−/−		
Integrin	Virgin	Pd15	Ld6	Virgin	Pd15	Ld6
α2	++	+	++	−	−	−
α6	+/−	+/−	+/−	++	+/−	+/−

The phenotype of the integrin α2−/− mice suggested a supportive rather than an essential role for integrin α2 in mediating collagen interactions, leaving open the possibility that other collagen or laminin receptors promote tissue morphogenesis (21). High levels of integrin α2β1 (collagen/laminin receptor) have been found on epithelial cells from benign hyperplastic/neoplastic mammary glands, but they are very reduced or lost on malignant cells of breast carcinoma. The loss of the integrin α2β1 expression and the increased invasiveness of neoplastic cells correlates with estrogen and progesterone receptor negativity (22). The promoter region of integrin α2 contains six Sp1-binding sites, consensus-binding sites for AP-1 and -2 complexes, and two potential estrogen responsive elements (EREs) (23). The presence of EREs means that the gene can be regulated by estrogen, and the presence of SP1 sites means that ERα and -β can have opposite effects on its regulation.

The increase in integrin α6 expression in ERβ−/− mice might reflect attempts of the cell to compensate for loss of integrin α2. The α6 promotor contains a putative glucocorticoid/progesterone receptor responsive element, together with AP-1 and c-myc-binding sites (24).

In the mammary gland, both secretory and myoepithelial cells are thought to arise from a multipotent tissue-specific epithelial stem cell, giving rise to all of the epithelial cells of a fully developed lactating gland, i.e., myoepithelial, ductal, and lobule-committed epithelial cells. The reduced myoepithelial cell layer in the lactating ERβ−/− mice suggests that ERβ regulates some signaling pathway essential for the differentiation of the myoepithelial cell lineage, probably involving integrin receptors and components of the underlying basement membrane.

Many of the changes seen in ERβ−/− mice are reminiscent of changes seen in breast cancer. *In vitro* evidence suggests that in mammary epithelial cells, oncogenes may be upstream regulators of both the expression and function of the morphoregulators E-cadherin and integrin α2β1 (25). Cx-32-gap junctions

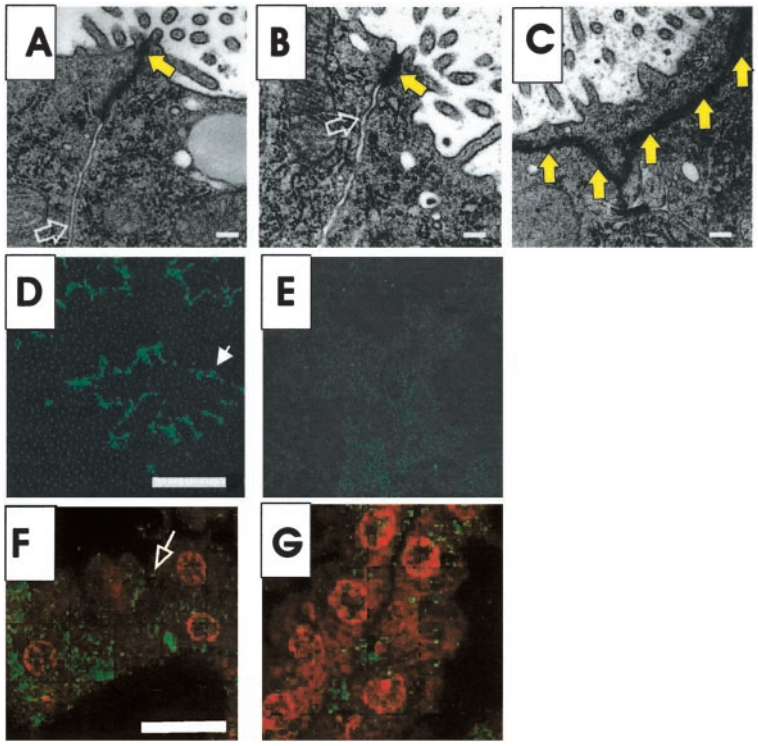

Fig. 4. Expression of tight junction and gap junction proteins. (*A–C*) Micrograph of the apical and lateral part of mammary luminal epithelial cells. The tight junction shows normal morphology and a clear cell border (open arrows) in WT mouse (*A*). In some ER$\beta-/-$ mice, there is a wider intercellular space (yellow arrows) with irregular small contact points (*B*). In ER$\beta-/-$ mice, a large complex of the irregular contact points was frequently found (*C*). (Bar = 200 nm.) (*D* and *E*) Distribution of occludin in alveoli from mammary glands of WT (*D*) and ER$\beta-/-$ mice (*E*) on day 6 of lactation. (Bar = 20 μm.) (*F* and *G*) Double immunofluorescence labeling of mammary glands of WT (*F*) and ER$\beta-/-$ (*G*) mice on day 6 of lactation. Nuclei are indicated by propidium iodide positivity (red), and the Cx-32 gap junctions in the luminal cells are labeled with fluorescein (green, arrow). At $\times$63, used to evaluate Cx expression, each fluorescent spot indicates a gap junction plaque.

form in mammary epithelium after parturition and lend characteristic properties to the epithelium: polarity, intercellular signaling, and mechanical continuity (26). Gap junction genes encoding Cx-26 and -43 suppress the cancer phenotype of human mammary carcinoma cells and play a role in growth control *in vivo* (27). A role for Cx-32 in cellular homeostasis and the development of the cancer phenotype has not been addressed so far, because its expression is not detectable before differentiation of mammary epithelial cells during lactogenesis II (28).

Instead of standard size tight junctions, irregular contact points of abnormal shape and length were detected in luminal epithelial cells of ER$\beta-/-$ mice. Because occludin could not be detected in these glands, these contact surfaces might consist of the other tight junctional protein, claudin (29). A proper size, shape, and composition of tight junctions is essential for controlling permeability of endothelial and epithelial cells. Changes in tight junction function have been shown to be an early and key aspect in cancer metastasis (30).

Cadherins are involved in signaling, cellular proliferation, and differentiation. Down-regulation of cadherin expression is associated with the invasiveness of tumor cells (31). The cytoplasmic tails of cadherins associate with and dynamically regulate the free β-catenin pool (32). β-Catenin regulates the function of the lymphocyte enhancer-binding factor/transcription enhancer factor (LEF/TEF) family of transcription factors and influences the transcription of genes like c-myc, cyclin-D1, matrilysin, c-jun, and c-fra (32). β-Catenin expression is increased on the membranes of mammary luminal epithelial cells in ER$\beta-/-$ mice. Such dysregulation might partly explain the observed aberrant expression of the proliferation marker Ki-67 in the mammary gland during lactation in ER$\beta-/-$ mice.

Although ovarian and pituitary hormones are known to be required for both ductal proliferation and lobuloalveolar differentiation of the mammary gland (18), the contribution of individual hormones to specific developmental processes and the mechanisms by which these hormones trigger morphogenesis are still poorly understood. Full-term pregnancy early in reproductive life is protective against breast cancer in women. Pregnancy also provides protection in animals against carcinogen-induced breast cancer, and this protection can be mimicked by administration of estrogen and progesterone (33). The mechanistic basis for this protective effect has not been elucidated. The data in the present study suggest that ERβ is essential for the fully differentiated phenotype of the mammary gland and, by contributing to cellular differentiation, homeostasis, and growth control, it may participate in the protection offered by pregnancy and lactation against breast cancer.

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1. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M. & Gustafsson, J.-Å. (2001) *Physiol. Rev.* **81,** 1535–1565.
2. Zhu, Y., Bian, Z., Lu, P., Karas, R. H., Bao, L., Cox, D., Hodgin, J., Shaul, P. W., Thoren, P., Smithies, O., *et al.* (2002) *Science* **295,** 505–508.
3. Wang, L., Andersson, S., Warner, M. & Gustafsson, J.-Å. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2792–2796.
4. Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P. & Mark, M. (2000) *Development (Cambridge, U.K.)* **127,** 4277–4291.
5. Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F.,

Sar, M., Korach, K. S., Gustafsson, J.-Å. & Smithies, O. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15677–15682.
6. Cheng, G., Weihua, Z., Makinen, S., Makela, S., Saji, S., Warner, M., Gustafsson, J.-Å. & Hovatta, O. (2002) *Biol. Reprod.* **66,** 77–84.
7. Palmieri, C., Cheng, G. J., Saji, S., Zelada-Hedman, M., Warri, A., Weihua, Z., Van Noorden, S., Wahlstrom, T., Coombes, R. C., Warner, M., *et al.*(2002) *Endocr. Relat. Cancer* **9,** 1–13.
8. Fisher, C. R., Graves, K. H., Parlow, A. F. & Simpson, E. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6965–6970.
9. Korach, K. S., Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Kimbro, K. S., Eddy, E. M., Migliaccio, S., Snedeker, S. M., Lubahn, D. B., *et al.* (1996) *Recent Prog. Horm. Res.* **51,** 159–186.
10. Gustafsson, J.-Å. (1999) *J. Endocrinol.* **163,** 379–383.
11. Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M. & Gustafsson, J.-Å. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 337–342.
12. Windahl, S. H., Vidal, O., Andersson, G., Gustafsson, J.-Å. & Ohlsson, C. (1999) *J. Clin. Invest.* **104,** 895–901.
13. Rasmussen, S. B., Young, L. J. T. & Smith, G. H. (2000) in *Methods in Mammary Gland Biology and Breast Cancer Res.*, eds. Margot, P. I. & Bonnie, B. A. (Kluwer/Plenum, New York), pp. 75–85.
14. Becker, D. L., Evans, W. H., Green, C. R. & Warner, A. (1995) *J. Cell Sci.* **108,** 1455–1467.
15. Couse, J. F. & Korach, K. S. (1999) *Endocr. Rev.* **20,** 358–417.
16. Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U. & Stein, H. (1984) *J. Immunol.* **133,** 1710–1715.
17. Nguyen, D. A. & Neville, M. C. (1998) *J. Mammary Gland Biol. Neoplasia* **3,** 233–246.
18. Daniel, C. W. & Silberstein, G. B. (1987) in *The Mammary Gland*, eds. Neville, M. C. & Daniel, C. W. (Plenum, New York), pp. 3–36.
19. Colognato, H. & Yurchenco, P. D. (2000) *Dev. Dyn.* **218,** 213–234.
20. Streuli, C. H. & Edwards, G. M. (1998) *J. Mammary Gland Biol. Neoplasia* **3,** 151–163.
21. Holtkotter, O., Nieswandt, B., Smyth, N., Muller, W., Hafner, M., Schulte, V., Krieg, T. & Eckes, B. (2002) *J. Biol. Chem.* **277,** 10789–10794.
22. Lanzafame, S., Emmanuele, C. & Torrisi, A. (1996) *Pathol. Res. Pract.* **192,** 1031–1038.
23. Zutter, M. M., Santoro, S. A., Painter, A. S., Tsung, Y. L. & Gafford, A. (1994) *J. Biol. Chem.* **269,** 463–469.
24. Nishida, K., Kitazawa, R., Mizuno, K., Maeda, S. & Kitazawa, S. (1997) *Biochem. Biophys. Res. Commun.* **241,** 258–263.
25. Takeichi, M. (1988) *Development (Cambridge, U.K.)* **102,** 639–655.
26. Loewenstein, W. R. (1990) *Am. Rev. Respir. Dis.* **142,** S48–S53.
27. Jansen, L. A., Mesnil, M. & Jongen, W. M. (1996) *Carcinogenesis* **17,** 1527–1531.
28. Locke, D., Perusinghe, N., Newman, T., Jayatilake, H., Evans, W. H. & Monaghan, P. (2000) *J. Cell. Physiol.* **183,** 228–237.
29. Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T. & Tsukita, S. (1998) *J. Cell Biol.* **141,** 397–408.
30. Martin, T. A. & Jiang, W. G. (2001) *Histol. Histopathol.* **16,** 1183–1195.
31. Hajra, K. M. & Fearon, E. R. (2002) *Genes Chromosomes Cancer* **34,** 255–268.
32. Gumbiner, B. M. (1995) *Curr. Opin. Cell Biol.* **7,** 634–640.
33. Medina, D., Sivaraman, L., Hilsenbeck, S. G., Conneely, O., Ginger, M., Rosen, J. & Omalle, B. W. (2001) *Ann. N.Y. Acad. Sci.* **952,** 23–35.